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FLUORIDE AND HEALTH

Epidemiological studies of fluoride exposure and hip fracture, myocardial infarction and osteosarcoma

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FLUORIDE AND HEALTH

EPIDEMIOLOGICAL STUDIES OF FLUORIDE AND HIP FRACTURE, MYOCARDIAL INFARCTION AND OSTEOSARCOMA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The aim of this thesis was to investigate the association between drinking water fluoride exposure and risk of hip fracture, myocardial infarction, and osteosarcoma. Swedish nation-wide population-based registers have been used throughout the thesis.

The risk of hip fracture was addressed in a population-based cohort of 452,824 eligible individuals with an estimated exposure to the same drinking water source from birth upon start of follow-up, (*i.e.* living in their municipality of birth). Information on residence from parish records was used to address a community water supplier for each individual in the cohort. The drinking water fluoride levels in our cohort ranged between <0.1 and 2.7 mg/L. We linked the Total Population Register, the National Patient Register, and the Swedish Death Register. We studied the association between drinking water fluoride level and risk of hip fracture. We found no association between fluoride exposure level and risk of hip fracture (compared to the very low exposure group, adjusted Hazard Ratio (aHR) for the low exposure group was 0.97; 95% CI = 0.94-0.99, aHR for the medium exposure group was 0.97; 95% CI = 0.94-1.00, and aHR for the highest exposure group was 0.98; 95% CI 0.93-1.04). Nor did we find an association between fluoride level and the risk of osteoporotic (low-trauma) hip fracture. Stratified analyses suggested that fluoride exposure in individuals younger than 80 years of age was associated with a decreased risk for hip fracture. However, no clear exposure-response effect was observed. We cannot rule out that unmeasured confounding may have influenced the observed results.

The risk of myocardial infarction was addressed in a population-based cohort of 455,619 eligible individuals with an estimated exposure to the same drinking water source from birth upon start of follow-up, (*i.e.* living in their municipality of birth). The fluoride exposure assessments and the retrieval of register data were performed in a similar manner as in study I (described above). The drinking water fluoride levels in our cohort ranged between <0.1 and 2.7 mg/L. We studied the association between drinking water fluoride level and risk of myocardial infarction. We found no association between fluoride exposure level and risk of myocardial infarction (compared to the very low exposure group, aHR for the low exposure group was 1.00; 95% CI 0.99-1.02, aHR for the medium exposure group was 1.02; 95% CI 0.99-1.04, and aHR for the highest exposure group was 1.01; 95% CI 0.98-1.04). Additional analyses was performed, looking at fatal and non-fatal myocardial infarction. No association was found. We found some evidence of a positive association between fluoride exposure level and risk of myocardial infarction in the northern part of Sweden (compared to the very low exposure group, aHR for the low exposure group was 1.04; 95% CI 1.00-1.07, aHR for the medium exposure group was 1.12; 95% CI 1.07-1.16, and aHR for the highest exposure group was 1.09; 95% CI 1.02-1.17). This association may in part be explained by unmeasured confounding. Moreover, only a small effect size is detected, and in combination with the

large sample size and total number of events, we consider that this finding is unlikely to be of significance.

The risk of osteosarcoma was addressed in a population-based case-control study consisting of 363 eligible osteosarcoma cases identified in the Swedish Cancer Register, and 1,815 control subjects randomly selected from the Total Population Register, and matched to cases (5:1) on birth year and sex. A control had to be alive and without an osteosarcoma diagnosis at the time of selection. All domestic movements and migrations (dates and locations) were collected for all cases and controls, from birth upon index date (date for cancer diagnosis). Information on residence was used to address a community water supplier for each individual in the cohort. The drinking water fluoride levels in our cohort ranged between 0.03 and 2.75 mg/L (ppm). We linked the Swedish Cancer Register, the National Patient Register, the Total Population Register, and The Register of Population and Population Changes. We studied the association between accumulated drinking water fluoride exposure and risk of osteosarcoma. Different exposure-times before osteosarcoma diagnosis were studied, as well as different exposure-ages, to evaluated potential important time-windows for exposure. We found no association between drinking water fluoride exposure and risk of osteosarcoma (aOR 0.99; 95% CI 0.67-1.16). However, this study has a few important limitations. We lack information on potential confounders such as additional socioeconomic variables and height at diagnosis. Moreover, misclassification of exposure when we only assess fluoride exposure from drinking water, other dietary source for fluoride (food, beverages, and dental hygiene products) might account for an important part of the total fluoride load. And additionally, non-differential misclassification of exposure (*i.e.* individuals categorized as exposed could in fact be unexposed and *vice versa*). We cannot rule out that the above mentioned methodological limitations may have influenced the results.

In conclusion, fluoride exposure from municipal drinking water does not appear to be associated with increased risk for hip fracture, myocardial infarction, or osteosarcoma in Sweden. This is in agreement with most findings from previous studies assessing similar drinking water fluoride exposure levels. The results of this thesis add to the body of evidence that ingestion of lower fluoride concentrations (<4 mg/L) is not associated with increased risk of adverse health effects.

LIST OF SCIENTIFIC PAPERS

This thesis is based upon the following paper, which will be referred to by their Roman numerals:

I. **Näsman P**, Ekstrand J, Granath F, Ekbom A, Fored CM.
Estimated drinking water fluoride exposure and risk of hip fracture: a cohort study.
J Dent Res. 2013 92(11):1029-34.

II. **Näsman P**, Granath F, Ekstrand J, Ekbom A, Sandborgh-Englund G, Fored CM.
Natural fluoride in drinking water and myocardial infarction: a cohort study in Sweden.
Science of the Total Environment. 2016 562:305-11.

III. **Näsman P**, Granath F, Ekstrand J, Ekbom A, Sandborgh Englund G, Naimi-Akbar A, Fored CM.
Natural fluoride in drinking water and osteosarcoma: a case-control study in Sweden.
Submitted manuscript.

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LIST OF ABBREVIATIONS

aHR	Adjusted Hazard Ratio
aOR	Adjusted Odds Ratio
CI	Confidence Interval
HR	Hazard Ratio
ICD	International Classification of Diseases
MI	Myocardial Infarction
OR	Odds Ratio
UL	Upper Tolerable Intake Level
WHO	World Health Organization
°dH	German Hardness Degree

1 INTRODUCTION

The use of fluoride in caries prevention is regarded as one of the greatest interventions in medicine[1], but its appropriateness has been intensely debated throughout the world. Opponents have raised doubts about safety, ethics, adverse health effects, and effectiveness of fluoride on oral health.[2]

The presence of fluoride in the caries prevention regime is a necessity, and large populations are exposed daily via drinking water, food (such as table salt and milk), or dental hygiene products. The benefits of topical fluorides (presented in the oral cavity), and low intake of fluoride (via drinking water for example) for dental caries prevention are firmly established; however, during childhood years slightly higher levels can lead to dental fluorosis, a mineralization disorder of the dental enamel.[2-4] Prolonged fluoride intake, at any age, may result in skeletal fluorosis, a condition with increased bone density and brittleness, with a potential increase in fracture risk.[2,3,5] In severe cases, where the ingested fluoride dose is very high, calcifications of ligaments and bone deformations may develop and cripple the individual.[5] Other adverse health effects such as cancer (osteosarcoma in particular), effects on the thyroid, and effects on the developing brain have been reported to be associated with ingested fluoride. However, despite the extensive research conducted over the last 50 years, the quality of the evidence of both the beneficial and harmful effects of fluoride is surprisingly poor. Studies often lack the appropriate study design and statistical methodology.

In this thesis we have used register data from national health and census registries, and historical drinking water data, to investigate the association between drinking water fluoride and the risk for hip fracture, myocardial infarction, and osteosarcoma. In the study design chosen and in the applied statistical analyses, we tried to avoid some of the shortcomings of the previous studies.

2 BACKGROUND

Fluoride is a halogen and the 13th most common element in the earth's crust, and thereby found in literally all living and non-living things in a broad range of concentrations.[6] The concentration of fluoride in water and food is dependent on geological conditions (such as soil and rainfall), and large geographical variations are seen.

Today over 377 million people in 28 countries live in areas with artificially fluoridated drinking water (for caries prevention), and a further 257 million people live in areas with naturally occurring fluoride levels.[7,8]

2.1 HISTORY OF FLUORIDE IN DENTISTRY

The link between oral health and fluoride dates back to the 1930s. In the late 1930s and 1940s both experimental and epidemiological studies identified the anti-cariogenic effect of fluoride.[9-13] Though, the discovery of fluorides anti-cariogenic effect was derived from observations in the early 20th century of mottled enamel (brown stains) in residents from Colorado Springs (USA).[14] Natural occurring fluoride in the drinking water was found to be the responsible cause for the enamel disturbance and discoloration, and subsequent named enamel fluorosis.[15-18] Studies on the prevalence of enamel fluorosis was investigated and when compared to the prevalence of dental caries an inverse relation was found.[9,11] Moreover, a dose-response pattern was observed between ingested fluoride from drinking water and dental fluorosis, water containing 1.0 mg/L of fluoride had a caries-protective effect with only very mild or mild forms of dental fluorosis.[10,12]

This discovery was followed by the hypothesis that adjustments of the drinking water fluoride levels to 1.0 to 1.2 mg/L could prevent dental caries. The hypothesis was tested in four pairs of cities in the USA and Canada, and resulted in a 50 to 70% caries incidence reduction in children, over a time period of 13-15 years.[19] After additional epidemiological studies on drinking water consumption in different geographical regions and climates the recommended level of fluoride in drinking water was lowered and set to 0.7 to 1.2 mg/L depending on climate (i.e. warmer climate equals to higher water consumption).[20] Water fluoridation was subsequently adapted in many countries where the fluoride level was considered deficient.

WHOs recommendations today are that artificially fluoridated drinking water should not exceed 1.0 mg/L, and that naturally occurring drinking water fluoride should not exceed 1.5 mg/L.[7] Recently, the US Department of Health and Human Services set the recommended level to 0.7 mg/L "the concentration that provides the best balance of protecting from dental caries while limiting the risk of dental fluorosis".[21]

In addition to adding fluoride to drinking water, other fluoride containing products (toothpaste, gels, tablets, mouth rinses, and food such as table salt and milk) were rapidly developed for dental caries prevention purpose. Initially it was believed that the systemic effect from fluoride on the developing enamel (preeruptively) prevented the development of dental caries. In the 1970s it was shown that the main effect from fluoride was due to its presence in the oral cavity.[22-24] Today, all caries preventive, and treatment methods using fluoride aim to supply fluoride to the oral environment (via drinking water, oral hygiene products, or food stuff), where it can pursue its effect on caries control.[22]

2.2 SOURCE OF FLUORIDE EXPOSURE

Human exposure to fluoride is mainly attributed to the diet (fluoride containing drinking water, other non-diary beverages, and food), and fluoride-containing oral hygiene products.[25] Though, fluoride is also found in air (dust, smoke) and chemicals (pesticides), but exposure from inhalation or dermal exposure are judged to be limited in industrialized countries, except for individuals having occupational exposure (industries).[2,26]

Drinking water

Drinking water is the most significant source of fluoride exposure, both historically and in modern times, according to large reports from Europe and the US.[2,27] Ground water has usually higher fluoride content than surface water (usually < 0.5 mg/L) and sea water (usually 1.2 to 1.5 mg/L).[6] The maximum concentrations of fluoride found in water are reaching 30 to 50 mg/L.[28] The natural fluoride concentrations found in European countries is generally believed to be low, but the geographical differences are large. According to EFSA there are no available systematic register data on drinking water content in European countries, but large variations are not only seen between different countries but also within countries.[25] When the Scientific Committee on Health and Environmental Risks conducted a critical review on human exposure to fluoride, they estimated that the mean value of the lowest fluoride concentration in European drinking water was 0.1 mg/L and the highest 3.0 mg/L.[27] In countries and geographical areas were the tap water is fluoridated (for cariostatic purposes) the concentration is around 1.0 mg/L. The tap water in Sweden is not artificially fluoridated, the fluoride found in drinking water is all naturally present. In some parts of Sweden the fluoride concentration is above the recommended limit for fluoride (above 1.5 mg/L), in particular private wells may have elevated fluoride concentrations.[29]

The level of exposure is not only dependent on the fluoride concentration in the drinking water, both the amount ingested and body weight are crucial.[30] According to European data on adult populations, the consumption pattern shows large differences; from 0.7 L/person/day

to 3.8 L/person/day across countries (median consumption 1.3 L/person/day).[25] The variance was attributed to human physiology and climate conditions. However, the exposure will mainly be dependent on the concentration of fluoride in water.[25,27] The WHO use 2.0 L/person/day as a default value for consumption.[7] Water and other liquid consumption data in children less than 12 years is estimated to under 0.5 L/person/day, and about 0.6 L/person/day for children aged 12 to 15 years.[25] However, the available data is sparse. The average consumption of tap water in Sweden (adult population) is estimated to be 1.8 L/person/day.[31]

Food

The second largest source of exposure is food. The fluoride level in fresh food is dependent on where it is processed and the water used, but also dependent on the water it is prepared in.[32] In Europe the fluoride content in food is generally low, between 0.02 to 0.29 mg/kg, but food stuff such as fluoridated table salt, fish, tea and bottled natural mineral water may contain higher fluoride concentrations.[25,33] The consumption of bottled water is limited in Sweden (0.06 L/day) compared to other European countries.[31]

Dental hygiene products

The concentration of fluoride in dental hygiene products vary depending on brand, and type of product. The upper level of fluoride concentration is 1,500 mg/kg (1.5%), higher concentrations may be prescribed for patients with high caries risk. In 2008, the mean consumption in Europe of fluoride containing toothpaste was 251 ml/year/person.[27] The ingestion of fluoride from dental hygiene products is depending on the fluoride concentration of the product, the amount used and swallowed.[27,34] The amount swallowed may account for an important part of the total fluoride load.

The recommended amount of toothpaste applied on the tooth brush for children is a "pea size" (about 0.25 g), and in adults the recommendations is the full length of the brush (about 0.75 g). In adults, about 10% of the amount of the toothpaste used on the brush is swallowed due to the spitting reflex, in children, under 8 years of age, the amount swallowed may be over 40% due to a less developed spitting reflex.[22] Eating/licking toothpaste is associated with a higher risk of fluorosis.[35] Additionally, the recommended fluoride concentration of the toothpaste is lower for children younger than 6 years of age (0.05 to 0.1% compared to 1.5% for older children and adults) due to the potential of swallowing.[27] The guidelines for children is also parental supervision and assistance when brushing to limit the fluoride ingestion. Studies have shown that if guidelines are followed (*i.e.* "pea size" amount, lower concentration of the paste, and parental supervision) the risk for dental fluorosis is reduced.[36,37]

2.3 THE FATE OF FLUORIDE

Ingested fluoride is rapidly, and nearly completely, absorbed from the gastrointestinal tract and distributed throughout the body.[32] Fluoride as hydrofluoric acid (HF) passes cell membranes, and plasma fluoride levels are detected within minutes after ingestion, with a peak level during the first hour.[30] The uptake is reduced when ingested with calcium-rich liquids or foods.[38,39] However, no difference is found between hard and soft drinking water, or between natural and artificially fluoridated drinking water, and the bioavailability of fluoride.[40-42] The fluoride found in feaces is believed to never have been absorbed from the gastrointestinal tract.[30]

Once fluoride is absorbed, it is cleared from the plasma through the excretion in urine, and to some extent in saliva and sweat, and by the uptake in calcified tissues (Figure 1).[32,43,44] About 50% of the absorbed amount is excreted via urine during the following 24 hours, most of the remaining amount is incorporated into calcified tissue. Since fluoride is a keen hard tissue seeker, about 99% of the body burden of fluoride is found in calcified tissues such as bone (where fluoride ions replace hydroxyl ions in hydroxyapatite crystals), and teeth (during preeruptive tooth development).[32] Besides bone and teeth, fluoride is also found in the pineal gland, another calcifying organ.[45]

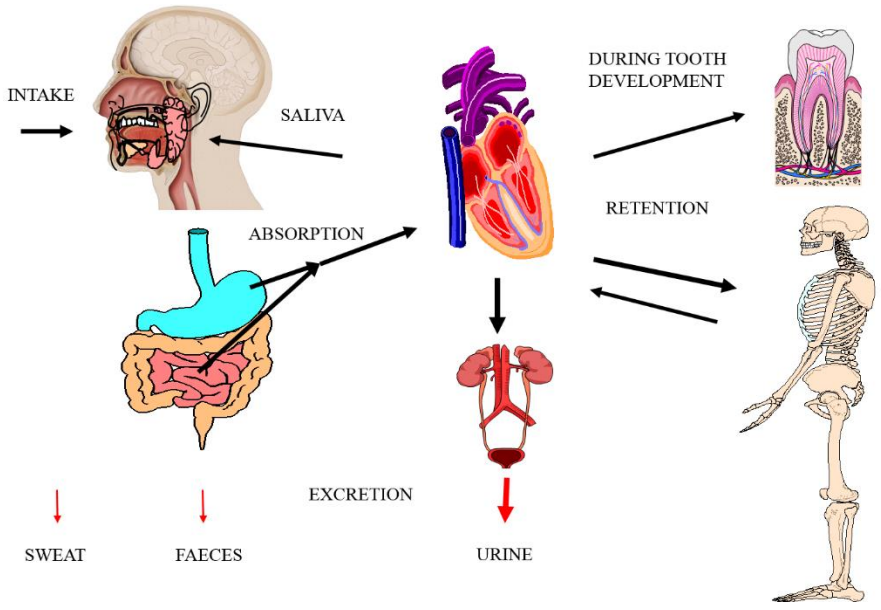


Figure 1. A humble illustration of the fate of fluoride.

Fluoride is poorly transferred from plasma to breast milk[46], but fluoride crosses via the placenta to the fetus, as well as over the blood-brain barrier. The fluoride level in plasma of the fetus is the same as in the mother, and fluoride concentrations in brain and fatty tissue are

thought to be about 20% of plasma concentration or less.[32,47] In most soft tissue the concentration of fluoride is the same as in plasma, except for the kidney where the concentration is high due to renal handling and elimination of fluoride.[32]

2.4 THE ACTION OF FLUORIDE

The debate on fluoride and its use in dentistry mainly questions the potential adverse health effects from ingested fluoride.[21] The suggested health effects have been many over the years. I will address the following; dental and skeletal fluorosis, bone fractures, cancer, cardiovascular disease, and neurological disease.

Dental effects

The presence of fluoride ions in the oral cavity (i.e. saliva or biofilm fluid) chemically reduce the rate of dental hard tissue demineralization during the caries process, enhance the remineralization process of the demineralized tissue, and reduce acidic production by the bacteria in the plaque.[48,49]

If fluoride is present during enamel formation, from the second fetal month to about 7-8 years of age[50], the fluoride ion is incorporated into the tooth enamel forming fluoroapatite.[51] Creating a less soluble structure of the enamel, but the preeruptive effect from fluoride on caries prevention is limited.[52] If excessive concentration of fluoride is present during this period in life when enamel formation takes place, enamel fluorosis may occur. The enamel disturbance can range from mild discolorations of the enamel to severe pitting and staining.[53] The process of mineralizing dental tissue disturbance is not yet fully understood[54], and other environmental and genetic factors may influence the development of dental fluorosis.[55,56]

The prevalence of enamel fluorosis in populations receiving drinking water with a fluoride concentration of 1.0 mg/L is about 48%.[10,57] The use of fluoride in young children is a risk-benefit balance, and both caries disease and enamel fluorosis are health concerns. To consider all forms of enamel fluorosis as adverse health effect has been the subject of debate. It has been judged to be a cosmetic effect, and in severe cases to be aesthetically displeasing.[2,26] This is largely based on that there is no evidence that fluorosis is associated with tooth loss, social, or psychological problems.[2,21,26,58]

Bone effects

As mentioned before, fluoride accumulates in calcified tissues throughout life. The retention during skeletal growth is higher than during adulthood. In infants, about 90% of ingested

amount is retained.[47,59] Whereas in an adult situation, 50 % is retained and 50 % is excreted, a steady state is reached.[32,59,60] Besides fluorides ability to incorporate into calcified tissues, it has also been shown that fluoride have potent effects on bone cells, in particular stimulation of osteoblast proliferation.[61,62] Both bone quality and quantity is affected.[32] Populations with fluoridated water has been shown to have an increase in bone density.[63,64] Fluoride has therefore been used in prevention regimes for osteoporosis, but the long term effects on bone strength and fracture risk is ambiguous.[65-67]

A number of epidemiological studies have investigated the association between fluoride exposure at <4.0 mg/L and fracture risk. A recent meta-analysis concluded no definitive patterns of association between fluoride exposure and fracture of any sites.[57] Studies with long duration were found to be the only variable associated with fewer fractures (a protective effect from fluoride).[57] However, the quality of the studies was low to moderate, and significant heterogeneity was found ($p<0.001$) among studies. Moreover, studies on hip fracture were evaluated in a meta-analysis, and findings suggests that there is limited evidence that chronic fluoride exposure from drinking water is associated with hip fracture risk.[68] However, caution is needed in the interpretation of the results due to lack of homogeneity, unmeasured confounding, and exposure misclassification. Additionally, evidence support the conclusion that increase fracture rates are likely in populations exposed to lifetime drinking water fluoride levels above 4.0 mg/L, compared to levels around 1.0 mg/L.[2,69]

As with dental tissue, chronic excessive fluoride intake may also cause skeletal fluorosis, with increased bone density, calcifications of ligaments and osteosclerosis, with different levels of severity and subsequent increased fracture risk.[2] There are limited data on skeletal fluorosis at drinking water fluoride levels <4.0 mg/L, and the National Research Council conclude that skeletal fluorosis is a rare condition at fluoride levels of 4.0 mg/L, but the relationship between ingestion, concentrations found in bone, and level of skeletal fluorosis needs more clarifications before any conclusions can be drawn.[2]

Bone cancer has also been focus of concern due to fluorides accumulation in bone and the ability to stimulate osteoblast proliferation.[2,32] Animal studies have found increased osteosarcoma incidence in male rats exposed to high fluoride dose.[70] Two recent systematic reviews evaluating human epidemiological studies on the association between fluoride exposure and cancer risk, reported no clear association.[57,69] Fluoride exposure at specific time windows (ages), has recently been suggested by Bassin et al. (2006)[71] to be of importance. The authors found an increased risk for osteosarcoma in boys exposed to fluoride at 6 to 8 years of age (with a peak at 7 years of age, aOR 5.46; 95% CI 1.50 – 19.90), but the need for caution in the interpretation of the findings was stressed by the authors, due to methodological limitations.[71,72] In a subsequent study, where the bone fluoride concentration was investigated, (instead of fluoride levels in the drinking water), the findings from Bassin et al.[71] could not be confirmed.[73] Despite the general scientific agreement that there is no

evidence of any association between ingested fluoride and increased osteosarcoma risk[73-83], the debate continuous.

Cardiovascular effects

In the 1960s and -70s, fluoride was suggested to attribute to the decline in cardiovascular death rates observed in the USA and other countries.[63,84,85] There were laboratory data[86-88] which suggested that fluoride could have an ability to inhibit soft tissue calcification, and additional findings of an association was reported from epidemiological data.[63,84,89] Recent studies in fluoride endemic areas have demonstrated the opposite; that excessive fluoride exposure affect the elastic properties of ascending aorta and moreover contribute to cardiac dysfunction.[90,91] According to the authors, the possible mechanism behind the findings was fluorides toxicological ability to induced oxidative stress and inflammation.[92,93] But the mechanism behind the impaired elastic properties of aorta is not fully understood. Another study of excessive fluoride exposure, found significant associations with carotid atherosclerosis development. The mechanism was attributed to decreasing levels of glutathione peroxidase by excessive fluoride concentrations, causing systemic inflammation and endothelial activation.[94,95]

Epidemiological data on fluoride exposure levels <2 mg/L have demonstrated that very low drinking water fluoride levels were associated with an increased risk of myocardial infarction.[89,96-98] But the potential protective effect found on myocardial infarction, is suggested to attribute fluorides impact on oral health improvement, *i.e.* via an indirect association.[98,99] Chronic low-grade bacterial infections, such as periodontitis and dental caries, have been linked to atherosclerosis and cardiovascular disease.[99-101]

Neurobehavioral effects

There are limited data on neurobehavioral effects of fluoride in humans. Studies on developmental effects of fluoride have mostly been conducted in China. A meta-analysis of 16 Chinese studies were published by Tang et al. (2008)[102], evaluating the influence of fluoride on children's IQ. High incidence of fluorosis and high fluoride air levels were associated with a higher odds of developing a low IQ, compared to low fluorosis areas. However, the analysis does not follow the praxis of a meta-analysis. Additionally, the included studies suffered from confounding, and suboptimal control over water quality. A previous report by the Scientific Committee on Health and Environmental Risks stated that the biological link for an association between fluoride and IQ has not been established.[27] Grandejean and Landrigan (2014)[103] published a review article in Lancet Neurology stating children exposed to fluoride had a decrement in IQ.[103] This statement was based on studies of low quality, however the impact in social media was high. A recent study from New Zealand could not confirm the findings from China. No association was found between fluoride

exposure (from drinking water, food, and dental hygiene products) and difference in IQ.[104] Adjustments were made for potential confounding factors such as age, sex, socioeconomic status, breastfeeding, and birth weight. The exposure levels investigated in the study is more relevant to exposure levels found in industrialized countries, and the findings are more generalizable to other populations.

2.5 HUMAN EXPOSURE

Exposure to fluoride vary substantially between individuals as well as between populations, dependent on drinking water source, dietary habits, and dental hygiene regimes. The intense debate over fluoride and its use in dentistry has led to several scientific investigations on fluoride and its hazard profile.[2,25,26,105,106] In 1986 the U.S. Environmental Protection Agency (EPA) established exposure standards for contaminants in the public drinking water.[2] Contaminants that may cause adverse health effects in humans, and fluoride is one of them.

Exposure standards that are established include the maximum contaminant level goal (MCLG). This is a health goal set at "concentrations at which no adverse health effects are expected to occur and the margins of safety are judged adequate".[2] A secondary maximum contaminant level (SMCL) is set for some contaminants, (fluoride is one of them), which is a guideline for "managing drinking water for aesthetic, cosmetic, or technical effects".[2] The MCLG for fluoride is set to 4.0 mg/L, and the SMCL to 2.0 mg/L.[2] The standards were set in 1986, and have been reevaluated repeatedly but not changed.[3]

Besides the standards for maximum concentration of fluoride in drinking water, the European Food and Safety Authority (EFSA) recently published a report on tolerable upper intake (UL) of vitamins and minerals in Europe.[106] The upper tolerable intake for fluoride is based on the risk for dental fluorosis (younger children), and the risk for bone fractures (older children and adults).[10,25] The UL for adults and children aged 15 years and older is 7 mg/day, and for children age 9 to 14 years 5mg/day. This was based on an UL of 0.12 mg fluoride/kg body weight/day. The UL for younger children, age 4 to 8 years is 2.5 mg/day and 1.5 mg/day for children age 1 to 3 years, based on an UL of 0.1 mg fluoride/kg body weight/day. An UL for infants (0 to 12 months of age) is not established by the EFSA.[106] A safe intake of fluoride is 0.22 mg/kg body weight/day according to UK Department of Health (1991), but recommendations from the US Institute of Medicine (1997) is 0.1 mg fluoride/kg body weight/day.[107,108]

Table 1. Upper tolerable intake level (UL) for fluoride, assessed by the European Food and Safety Authority (EFSA).

Age	Fluoride/day
Adults and children > 15 years of age	7 mg
Children 9-14 years of age	5 mg
Children 4-8 years of age	2.5 mg
Children 1-3 years of age	1.5 mg
Children <1 year of age	0.10 - 0.22 mg/kg body weight

Estimated exposure in adults

The estimated daily fluoride exposure in Europe from drinking water may correspond to amounts between 0.13 to 8.40 mg/day in adults and children aged 15 years and older.[27] The estimated daily fluoride exposure in Europe from food and non-diary beverages correspond to 0.120 mg/day in adults. Noteworthy is that the addition from fluoride supplemented food (such as table salt) is estimated in adults to be 0.25 mg per day.[27] The estimated daily fluoride exposure from ingested toothpaste may correspond to amounts between 0.025 and 0.225 mg/day when brushing twice a day.[27] The ingested amount is depending on the fluoride concentration of the product, the amount used and swallowed.

In European conditions it is estimated that the total fluoride exposure only would exceed the UL if around 3.0 L of drinking water with the highest fluoride concentration (~3.0 mg/L) is consumed daily.

Estimated exposure in children

The estimated daily fluoride exposure in Europe from drinking water may correspond to amounts between 0.05 to 4.5 mg/day in children aged 1 to 15.[27] The estimated daily fluoride exposure in Europe from food and non-diary beverages correspond to 0.114 mg in older children, and 0.042 mg in young children. In children older than 8 years of age the estimated daily fluoride exposure from ingested toothpaste may correspond to amounts between 0.025 and 0.225 mg/day when brushing twice a day, depending on the fluoride concentration of the product, the amount used and swallowed. In younger children, age 1 to 8 years, the estimated daily fluoride exposure from ingested toothpaste may correspond to amounts between 0.1 and 0.9 mg/day when brushing twice a day, where the worst case scenario is when "full length"

(0.75g) of highest concentration (1,500 mg/kg) of toothpaste is used and spitting is insufficient (swallowing 40%).[27]

For children 6 to 12 years of age, the total fluoride exposure is estimated to exceed the UL only if around 1.5 L of drinking water with the highest fluoride concentration (~3.0 mg/L) is consumed daily. For children 1 to 6 years of age, the UL is estimated to exceeded the UL if around 1.0 L of drinking water with fluoride concentration of ~0.8 mg/L is consumed daily, in combination with high consumption of toothpaste.[27]

Estimated exposure in infants

During the first months of life the main food source for infants is breast milk or formula. Therefore, the fluoride exposure mainly depends on if they are fully or partly breast fed. As mentioned before, the amount of fluoride found in human breast milk is low (~6.0 µg/L), this correspond to less than 0.001 mg/kg/day.[59] But if the child is formula-fed, the fluoride exposure is dependent on the water used, the brand, and the amount fed. The estimated daily fluoride exposure in formula fed infants may correspond to amounts between 0.02 and 0.47 mg/kg body weight/day.[27]

In infants being formula-fed, the risk for excess fluoride exposure is evident if the drinking water exceed 0.8 mg/L.

3 AIMS

The general aim of this thesis is to investigate possible adverse health effects from natural drinking water fluoride exposure, focusing on three different health outcomes: bone fracture, myocardial infarction, and malignant osteosarcoma.

Study I — Our aim was to investigate the association between fluoride exposure and risk of hip fracture in a cohort of individuals chronically exposed to natural fluoride in the drinking water.

Study II — Our aim was to investigate the association between fluoride exposure and risk of myocardial infarction in a cohort of individuals chronically exposed to natural fluoride in the drinking water.

Study III — Our aim was to investigate the association between fluoride exposure and risk of osteosarcoma.

4 METHODS

4.1 STUDY SETTING

In all studies in this thesis we used data from Swedish population-based national registries. All Swedish residents are assigned a unique personal identification number, at birth or upon immigration that allows linkage among national registries.[109] The Swedish health care system is public and hospital referrals are based on geographical residency rather than financial capacity or health insurance.

4.2 DATA SOURCES

4.2.1 The Total Population Register

The Total Population Register, is maintained by Statistics Sweden and updated daily. The register contains individual data such as personal identity number (or national registration number), age, sex, place of birth, citizenship, civil status, highest educational level (since 1985), date of death, and other demographic information of all residents in Sweden since 1961.[110] Migrations (both domestic and international) have been registered since 1969.

4.2.2 The Cause of Death Register

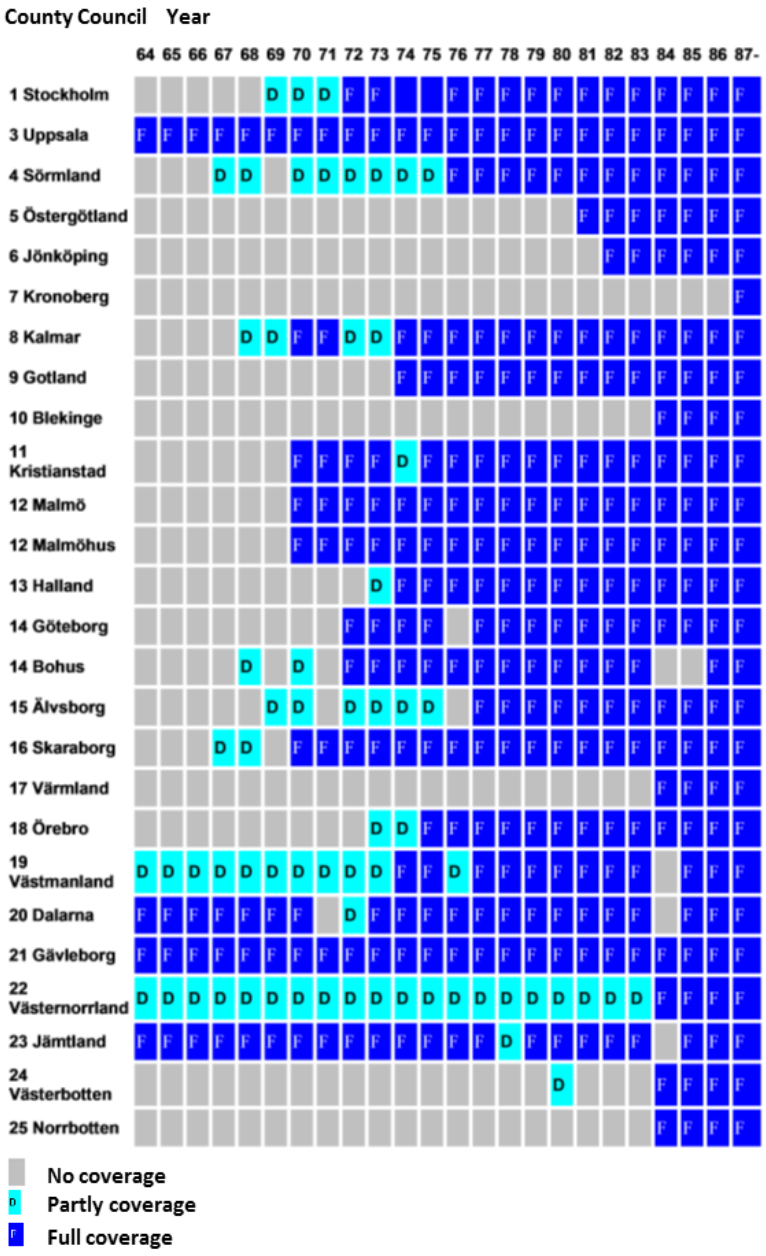
The Cause of Death Resister, which is managed by the National Board of Health and Welfare, contains data on the date of death and primary and contributing causes of death for all Swedish residents since 1961, regardless if the death occurred in Sweden or abroad.[111] There are no missing deaths since 1997, but in up to 0.5% of the deaths the cause of death is unknown.

4.2.3 The National Patient Register

The National Patient Register, which is managed by the National Board of Health and Welfare, contains data on hospitalizations (inpatient) from 1964. Initially the register contained data from only six Swedish counties, but gradually expanded and reached full national coverage in 1987 (Table 2).[112] The outpatient register was initiated in 2001 and includes data on diagnoses in non-primary outpatient care, coded according to ICD version 10. The coverage in the National Patient Register is close to 100% for the inpatient part, and approximately 87% for the outpatient visits (missing primarily from private care givers).[113,114]

The National Patient Register contains the patient's personal identification number, the date of hospital admission and discharge, and one primary discharge diagnosis, and up to seven additional diagnoses coded according to the International Classification of Diseases (ICD-7 until 1968, ICD-8 from 1968 through 1986, ICD-9 from 1987 to 1996, and ICD-10 thereafter).

Table 2. Coverage in the Swedish National Patient Register (inpatient part) by year and county council.[98]



4.2.4 The Swedish Cancer Register

The Swedish Cancer Register established in 1958, is managed by the National Board of Health and Welfare.[115] The register is a nationwide register based on mandatory reports of cancer diagnoses from healthcare providers in Sweden. The completeness and accuracy of the Swedish Cancer Register is generally high: over 96% of patients with a cancer diagnose are registered.[116]

4.3 STUDY DESIGN

Study I and II are population-based retrospective cohort studies (Table 3). In a retrospective cohort study, data collected in the past are used to identify the population of interest and the time period where they were at risk occurred before the study was conducted.[117] Typically, a cohort comprise of individuals classified according to exposure status, and then followed over time, and the occurrence of one or more specific outcomes (diseases) are measured. The aim is to compare the ratio between the disease rate of the exposed population and that of the unexposed population (or a subgroup of the individuals in the cohort).

Study III is a population-based case-control study (Table 3), where cases are identified and control subjects are sampled from the entire source population that gave rise to the cases.[117] The exposure status for cases and controls are determined. The aim is to yield a measure of association between an exposure and the outcome of interest (disease). An odds ratio is calculated, which is the ratio of the odds of an exposure in the case population to the odds of an exposure in control population. Case-control studies is preferred when studying a rare disease or outcome.

Table 3. Overview of studies included in the thesis.

	Study I	Study II	Study III
Design	Cohort	Cohort	Case-control
Population	All individuals born 1900-1919 in Sweden, alive and living in their municipality of birth at the start of follow-up	All individuals born 1900-1919 in Sweden, alive and living in their municipality of birth at the start of follow-up	All individuals diagnosed with osteosarcoma in Sweden, born January 1st, 1969 and onwards, and matched controls (5:1)
Number	452,824[1]	455,619[2]	2,178; 363 cases, 1,815 controls
Data sources	The Total Population Register The National Patient Register (inpatient) The Cause of Death Register	The Total Population Register The National Patient Register (inpatient) The Cause of Death Register	The Total Population Register The Swedish Cancer Register The National Patient Register (in- and outpatient)
Time of data collection	January 1964* - December 2006	January 1964* - December 2006	January 1969 - December 2014
Exposure	Drinking water fluoride level	Drinking water fluoride level	Drinking water fluoride level
Outcome	Hip fracture (Subanalyses on osteoporotic hip fracture)	Myocardial infarction (Subanalyses on fatal- and non-fatal myocardial infarction)	Osteosarcoma

[1] 21,393 individuals excluded from the base-line cohort due to hip fracture prior to start of follow-up (n=940) or missing exposure data (n=20,453).

[2] 18,598 individuals excluded from the base-line cohort due to myocardial infarction prior to start of follow-up (n=4,341) or missing exposure data (n=14,257).

* Start of follow-up from the year the county council of residence had full coverage in The National Patient Register.

4.3.1 Study I and II

Our study population were identified in the Total Population Register, and consisted of individuals born in Sweden between January 1st, 1900 and December 31st, 1919, alive and living in their birth parish at the time of start of follow-up. The follow-up started from the date the county of residence (birth) had full coverage in the National Patient Register, (*i.e.* earliest start January 1st, 1964 and latest start January 1st, 1987). Out of 1,951,244 individuals born between January 1st, 1900 and December 31st, 1919, 474,217 individuals were alive and living in their birth parish at the time of start of follow-up.

Study I

In study I the outcome of interest was first hip fracture (International Classification of Diseases (ICD)-7 to 9 codes: most diagnoses starting with 820; and ICD-10 codes: S720, S721, or S722). 940 individuals in the baseline cohort were diagnosed with a hip fracture prior to start of follow-up and were subsequently excluded. Additionally, 20,453 individuals had missing exposure data and were excluded from the analyses. Thus, the number of eligible individuals was 452,824.

Hip fracture was the failure event, and individuals with no hip fracture were censored at death, migration from the municipality of birth, or at the end of follow-up (December 31st, 2006), whichever occurred first. All eligible study subjects were followed through linkage to the Total Population Register to ascertain domestic movement, emigration, or death during follow-up. Hip fractures were determined from the National Patient Register and the Cause of Death Resister by the ICD-codes. Additional codes for low-trauma osteoporotic fractures were retrieved, *i.e.* a fracture occurring after a fall from less than standing height (ICD7: E903.9; ICD8-9: E885 and E886; and ICD-10: w00, w01, w03 and w18). All primary and additional discharge diagnoses, and primary and contributing causes of death diagnoses were examined for the first occurrence of the outcome of interest. High-energy trauma fractures were not included in the study.

Study II

In study II the outcome of interest was first myocardial infarction (International Classification of Diseases (ICD)-7 codes: 420.10:420.17, ICD-8 to 9 code: 410; and ICD-10 codes: I21-I22). 4341 individuals in the baseline cohort were diagnosed with a myocardial infarction prior to start of follow-up and were subsequently excluded. Additionally, 14,257 individuals had missing exposure data and were excluded from the analyses. Thus, the number of eligible individuals was 455,619.

Myocardial infarction was the failure event, and individuals with no myocardial infarction were censored at death, migration from the municipality of birth, or at the end of follow-up

(December 31st, 2006), whichever occurred first. All eligible study subjects were followed through linkage to the Total Population Register to ascertain domestic movement, emigration, or death during follow-up. Myocardial infarctions were determined from the National Patient Register and the Cause of Death Resister by the ICD-codes. All primary and additional discharge diagnoses, and primary and contributing causes of death diagnoses were examined for the first occurrence of the outcome of interest. Information on whether the myocardial infarction was fatal or not (death within 28 days from hospitalization / the acute event) was also retrieved from the Cause of Death Register.

4.3.2 Study III

A case was defined as a histologically verified diagnosis of primary, malignant osteosarcoma (ICD-7: 196, PAD: 766). All individuals diagnosed with osteosarcoma in Sweden, and born January 1st, 1969 and onwards, were identified in the Swedish Cancer Register. 363 eligible osteosarcoma cases were retrieved, only tumours of bone topography were included. Control subjects were matched to cases (5:1) on birth year and sex. 1,815 controls were randomly drawn from the Total Population Register at the year before the corresponding (matched) case was diagnosed. A control had to be alive and without a malignant bone cancer at the time of selection. The same control could be drawn as a control more than once, and cases were eligible to be drawn as controls.

Denominator data were retrieved from the Total Population Register provided by Statistics Sweden. Data on highest known educational level, domestic movements and migrations (dates and locations) were collected for all cases and controls, from birth upon index date (date of cancer diagnosis). For cases and controls all inpatient and outpatient data from birth upon index date were retrieved from The National Patient Register, and all cancer diagnosis were retrieved from the Swedish Cancer Register, from birth upon index date.

4.4 EXPOSURE ASSESSMENT

Information on community drinking water content was provided from the Swedish Water & Wastewater Association (SWWA) (available annually from 1960 to 1968, and from 1969 to 1999 every fifth year)[29], and from The Geology Survey of Sweden (SGU) (annually from 1999)[118], which collects and evaluates data from all Swedish water companies. Information about the water treatment plant (geographical location, type of water supply, type of distribution, produced water (m^3/day), number of affiliated households, etc.), and a number of different physical and chemical water properties for each water treatment plant was available.

Fluoride exposure was limited to the exposure from community water supplies. Drinking water data from private wells were not available, thus causing missing exposure data for

some of the individuals in the cohort. We do not have information on individual water intake, intake from other dietary sources of fluoride, or the use of fluoride containing dental hygiene products.

In Study I and II each water treatment plant was assigned a mean value of fluoride level (mg/L) in the distributed drinking water. The fluoride level in the municipalities of our study population ranged from <0.1 up to about 2.7 mg/L. The variation of fluoride levels in the investigated municipalities was small during the study period. A variation of more than ±0.2 mg/L was detected for only eight percent of the study population. Information on residence from parish records was used to address a community water supplier for each individual in the cohort. The fluoride distribution was divided into concentration categories relevant for drinking water fluoride concentrations for the prevention of dental caries: very low <0.3 mg/L, low 0.3 - <0.7 mg/L, medium 0.7 - <1.5 mg/L and high ≥1.5 mg/L.[7]

In Study I, 20,453, and Study II, 14,257, cohort subjects had missing exposure data, due to no community water supplier in the municipality of residence, or no measurement report.

Moreover in Study II the fluoride distribution was also divided into quantiles (octiles), eight equal-size groups. Additionally each water treatment plant was assigned a mean value of water hardness in the distributed drinking water. The total water hardness was measured in German hardness degrees, °dH (1 °dH = 7.1 mg calcium/100 ml water). The water hardness ranged between 2 and 147 mg/L in our study, and for about 2% of the cohort (n=9896) data on hardness was missing. The hardness distribution was divided into concentration categories as follows: very soft ≤2 °dH (≤15 mg/L), soft 2.1 – 4.9 °dH (15 – 35 mg/L), medium hard 5.0 – 9.8 °dH (36 – 70 mg/L), and hard ≥9.9 °dH (≥71 mg/L).

In Study III each case and control were assigned a mean drinking water fluoride level for each year from birth upon index date (date for osteosarcoma diagnosis). The fluoride level in the municipalities of our study population ranged from 0.03 up to about 2.75 mg/L. Fluoride was modelled as a continuous variable as well as an ordinal variable, dividing the distribution into quartiles and concentration categories relevant for drinking water fluoride concentration for the prevention of dental caries.[7] For 8.6% (n=188) of the population, residence information was missing at some time between birth and index date, which led to missing fluoride exposure data. Missing residence information was either due to emigration (8 controls), or born abroad (32 cases and 148 controls), about 35% had missing residence information of more than 50% of the years from birth upon index date.

4.4.1 Missing data

In this thesis missing exposure data was handled in two different ways. In Study I - III, individuals in the cohort having missing drinking water fluoride, or hardness levels were excluded from the analysis. We only focused on the individuals in the cohort having valid exposure data.

In Study III, a second approach was considered, were we assigned exposure values based on the individual's own exposure data during the study period.[119] Impute of either the lowest recorded exposure value, or the highest recorded exposure value. We additionally performed different models including only individuals having less than 25%, or less than 50% of the years missing from birth upon index date.

4.5 STATISTICAL METHODS

4.5.1 General aspects

We used the Statistical Analysis System (SAS) package, version 9.3 (Study I-II) and version 9.4 (Study III), for all statistical analyses.

4.5.2 Baseline characteristics

In Study I and II the categorical variables, presented as proportions, were assessed with Chi-square test, and the continuous variables, presented as median values, were compared with the non-parametric Kruskal-Wallis test. In Study III the characteristics of cases and controls were assessed by univariate conditional logistic regression, to determine any significant differences between the two groups.

4.5.3 Cox Proportional Hazards Regression Model

The Cox proportional Hazards Regression Model is commonly used in time-to-event analysis.[117] The Cox model is used to model the relationship between a set of covariates (one or more) and the hazard rate. The output is given as the hazard ratio (HR). The hazard is the risk of an event (outcome) in a specific time interval, assuming survival to that time. A hazard ratio of 1 correspond to equal risk compared to the comparator group. A hazard ratio below 1 correspond to a decreased risk, whereas a hazard ratio above 1 correspond to an increased risk. If the confidence intervals include 1 the hazard ratio is not statistically significant.

The basic aim of the Cox regression analyses in Study I was to investigate the association between fluoride exposure and hip fracture. Hip fracture was the failure event, and individuals with no hip fracture were censored at migration from the municipality of birth (n=12,562), death (n=370,564), or end of follow-up, December 31st, 2006 (n=29,378), whichever occurred first. Person-years were calculated for each individual in the cohort. Time was modeled from the date the county of residence had full coverage in the National Patient Register to the date of the failure event or the date of censoring. Crude hazard ratios

included sex, and attained age in 5-year categories. We adjusted for calendar period for study entry in 5-year categories, and geographical area of residence (county). Fluoride exposure was analyzed as a categorical variable (4 categories), we used the lowest exposure group (i.e. the largest exposure group) as reference. Analyses were stratified by sex, and attained age (<70 years, 70 - <80 years and ≥80 years of age). To test whether the effect of fluoride was modified by sex or attained age, Wald's heterogeneity test was performed. Additionally, we restricted the analyses to only the low trauma osteoporotic fractures in the cohort.

The basic aim of the Cox regression analyses in Study II was to investigate the association between fluoride exposure and myocardial infarction. Myocardial infarction was the failure event, and individuals with no myocardial infarction were censored at migration from the municipality of birth (n=11.670), death (n=214,970), or end of follow-up, December 31st, 2006 (n=24,450), whichever occurred first. Person-years were calculated for each individual in the cohort. Time was modeled from the date the county of residence had full coverage in the National Patient Register to the date of the failure event or the date of censoring. Crude hazard ratios included sex, and attained age in 1- and 5-year categories. We adjusted for calendar period for study entry in 1- and 5-year categories, geographical area of residence (county), and total water hardness (4 categories). Fluoride exposure was analyzed as a categorical variable (4 categories and quantiles), we used the lowest exposure group (i.e. the largest exposure group) as reference. Fluoride exposure was additionally analyzed as a continuous variable, implying a log-linear association. To test for a possible threshold effect a cut point of 1.0 mg/L was set. Analyses were stratified by sex, attained age (<70 years, 70 - <80 years and ≥80 years of age), geographical area of residence (south, middle, north), and time period (1964-1984, 1985-2006). To test whether the effect of fluoride was modified by sex, attained age, geographical area, or time period, Wald's heterogeneity test was performed. To avoid prevalent cases that could affect the result, a sensitivity analysis excluding the first year of follow-up were performed. Additionally, we stratified the analyses by fatal and non-fatal myocardial infarctions.

4.5.4 Logistic regression

Logistic regression is used to model the association between the dependent variable (a binary outcome, e.g. presence or absence of disease) and the independent variables (categorical and/or continuous).[117] The method of choice in case-control studies. The logistic regression model estimates odds ratios (OR), and is defined as the cases' odds of having been exposed to a risk factor, divided by the controls' odds of having been exposed to the same risk factor.

Conditional logistic regression is often used in matched case-control studies, where each individual is only compared with the matched individual/individuals.[117] Though, the effect of the matching variables is minimized. Unconditional multivariate logistic regression could also be used to calculate odds ratios, where all the matching variables are included in the analysis, but then an overestimate of the odds ratio will be obtained.

The basic aim of the conditional logistic regression analyses in Study III was to investigate the association between fluoride exposure and osteosarcoma in a matched case-control design. The dependent variable identifying cases (osteosarcoma diagnosis), and measures of fluoride exposure were the primary independent variables (examined both as a continuous as well as a categorical variable). Adjusted logistic regression models included education, other cancer and history of bone fractures prior to index date. Fluoride exposure was analyzed as a categorical variable (4 categories and quartiles), we used the lowest exposure group as reference. Fluoride exposure was additionally analyzed as a continuous variable, implying a log-linear association. To test for a possible threshold effect a cut point of 0.7 mg/L was set. The analyses was further stratified by age, sex, and time period for exposure. Additionally we performed sensitivity analyses where we used different handling of missing exposure data, and where we excluded individuals diagnosed with other cancer prior to index date.

4.6 ETHICAL CONSIDERATIONS

All studies (I-III) are register-based studies, based on information collected from patients in a standardized manner. Informed consent is not required for the inclusion of data in the national registers, but to ensure that the potential benefits for the population outweigh the risk for the individual, an ethical board have to evaluate the importance and soundness of the research[120], and an ethical approval can endorse the access to national register data for research purpose.

All studies (I-III) were approved by the Research Ethics Committee of the Karolinska Institutet, Stockholm, Sweden on the 12th of October, 2006 (dnr 2006/1052-31), and additions were approved by the same committee on the 9th of March, 2011 (dnr 2011/177-32). All data in this project were handled and analyzed at an aggregated level to protect patient privacy.

5 RESULTS

5.1 STUDY I

There was no significant association between drinking water fluoride exposure and hip fracture. Compared to the lowest exposure group (named very low) the hazard ratios were HR 0.97; 95% CI 0.94-0.99 for the low group, HR 0.97; 95% CI 0.94-1.00 for the medium group, and HR 0.98; 95% CI 0.93-1.04 for the high group. Adjusting for potential confounding factors such as age, sex, calendar period and geographical area did not change the risk estimates. Analysis were stratified by age (<70 years, 70 - <80 years, and ≥80 years) and sex. The risk estimates were statistically significantly different between age groups (p <0.001), and the results suggest a protective effect of fluoride in the two younger age groups. Though, the majority of fractures occurred above the age of 80 years. No difference was seen between men and women (p <0.540).

Additional analyses, looking specifically at hip fractures coded as low-trauma fractures (regarded as osteoporotic fractures), was performed in our population. No association was found between drinking water fluoride exposure and low-trauma fractures, the HRs did not differ from the overall HRs. Adjusting for potential confounding factors such as age, sex, calendar period and geographical area did not change the risk estimates. Analysis were stratified by age (<70 years, 70 - <80 years, and ≥80 years) and sex. The risk estimates were statistically significantly different between age groups (p <0.001), and the results suggest a protective effect of fluoride in the two younger age groups. Though, the majority of fractures occurred above the age of 80 years. No difference was seen between men and women (p <0.093).

5.2 STUDY II

There was no statistically significant association between drinking water fluoride exposure and myocardial infarction. When the exposure variable was analyzed as a categorical variable (4 levels), compared to the lowest exposure group (named very low) the hazard ratios were as follows: HR 0.99; 95% CI 0.98-1.00 for the low group, HR 1.01; 95% CI 0.99-1.03 for the medium group, and HR 0.98; 95% CI 0.96-1.01 for the high group. Adjusting for potential confounding factors such as attained age (1- and 5-year categories), sex, calendar period for study entry, geographical area and total water hardness (4 levels) did not change the risk estimates. No difference was seen either between men and women (p=0.470), nor between age groups (<65 years of age and ≥65 years of age) (p=0.437) or time periods (1964-1984 and 1985-2006) (p=0.058).

A geographical variation in the risk pattern was seen ($p<0.001$), the results suggest an association between fluoride exposure and myocardial infarction in the north of Sweden. Compared to the lowest exposure group (named very low) the hazard ratios were as follows: HR 1.04; 95% CI 1.00-1.07 for the low group, HR 1.12; 95% CI 1.07-1.16 for the medium group, and HR 1.09; 95% CI 1.02-1.17 for the high group.

Additional separate analyses was performed, looking at fatal and non-fatal myocardial infarctions. No association was found. Adjusting for potential confounding factors such as attained age (1- and 5-year categories), sex, calendar period for study entry, geographical area and total water hardness (4 levels) did not change the risk estimates.

5.3 STUDY III

Overall, no statistically significant association was found between fluoride exposure and osteosarcoma. Treating the exposure variable as a continuous variable, the odds ratio (OR) for osteosarcoma for 1 mg/L increase in drinking water fluoride level was 0.99 (95% CI 0.67-1.44). Adjustments were made for age, sex, level of education, other cancer or history of bone fractures prior to index date. Analyses restricted to individuals younger than 20 years of age did not change the result. No evidence of a threshold effect was seen, OR 1.04 (95% CI 0.76-1.43) with cut-off at 0.7mg/L.

Separate analyses were performed at specific time windows; cumulative fluoride exposure up to ages 2, 6, 12 and 16 years. No association was found. Additional analyses were performed for specific time periods prior to index date; 1, 2, 3, 4 and 5 years. No association was found. All analyses were also stratified by sex and age (younger than 20 years of age). No association was found.

Finally. Sensitivity analyses were performed. Missing values for fluoride was assigned (impute), the result did not change. Nor did the result change when excluding individuals diagnosed with other cancer prior to index date.

6 GENERAL DISCUSSION

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 Study design

Randomized clinical trials are considered the gold standard in evidence based medicine, whereas observational studies often are considered inferior.[121] However, observational studies are justified in medical research for several reasons.[122,123] Most importantly, experimental studies are not always possible, and may be inappropriate or unreasonable because of ethical and legal reasons. Cohort and case-control studies are the two main types of epidemiological studies. The choice of study design is generally a compromise between efficiency and validity, but a suboptimal study design may affect the validity and introduce bias that are difficult to remove in the statistical analysis. Yet in any research the choice of an eligible method for statistical analysis is essential, but the choice of an appropriate study design is many times even more important as the causal interpretation of the results often is dependent on the design of the study.[117]

6.1.1.1 Cohort studies (I-II)

Cohort studies are suitable for studies of multiple outcomes or rare exposures, and considered to have a higher validity than case-control studies.[117] However, for cohort studies to be efficient, a reasonable number of study subjects have to be followed to be able to study the disease of interest. They are considered time consuming and expensive, particularly in large prospective cohort studies and studies of rare diseases, but this is not the case in historical cohort studies based on national registers. Since the data is already collected and registered, the time consumption is substantially reduced and the costs are not dependent of the study size. Prospectively collected data, as in Swedish national registers, minimize the risk of recall bias, and additionally, when the collection of data is made without knowledge of future studies, the risk of biased registrations is limited. However, the disadvantage with historical data is that there might be a lack of information that limit access to potential confounding factors and exposure data.[117]

In study I and II we aimed to identify a population that had been living in the same geographical area (parish) since birth, and thereby have had the same source for exposure (drinking water). Since the outcomes of interest (hip fracture and myocardial infarction) occur relatively late in life, the study population had to be old enough for the outcome of interest. Therefore our study population was born between January 1st 1900 and December 31st 1919.

6.1.1.2 Case-control studies (III)

When studying rare outcomes, a case-control design may be the most suitable approach.[117] A cohort design would be inefficient and time consuming. In contrast to cohort studies, only a sample of the source population is collected in case-control studies, which generates risk for bias. The validity of the study depends on the comparability of the cases and controls. The source population is the set of individuals at risk for developing the outcome of interest, and both the cases and the corresponding controls should be representative of the same population.[124]

Controls can be matched on confounders to improve the efficiency, but only strong risk factors should be considered for matching. However, the matching does not control for confounding, the statistical analysis must adjust for the matching factors.[117] If the control subjects are too closely matched to their corresponding case they may not be representative of the general population and thereby affect the generalizability of the results. Additionally, if the matching variable is strongly associated with the exposure there is a risk for overmatching which may cause an underestimation of the true difference.

In study III we aimed to identify all individuals with an osteosarcoma diagnosis, and the individuals with osteosarcoma were matched to controls within the same source population. To assure complete residence history (i.e. complete history of exposure) the individuals had to be born 1st of January 1969 and onwards, since register data on domestic movements and migrations are available since the year 1969.

6.1.2 Internal validity

Internal validity is the extent to which the study actually measure what it is intended to do.[125] The internal validity is threatened by two types of error, systematic error (bias) and random error.

6.1.2.1 Selection bias

Selection bias is a systematic error resulting from the procedures used when selecting study subjects and from factors affecting the study participation.[117] In all present studies the study populations were retrieved from population based registers which are complete and continuously updated. Due to the population-based design, the probability of being sampled is the same for all Swedish residents and limits the selection bias. Though, the criteria for our study population in study I and II were restricted to individuals living in their municipality of birth by the time of start of follow-up, *i.e. non-movers*. We cannot rule out that *non-movers*

might differ from the general population, but that the exposure (drinking water fluoride) would be associated with domestic movements or emigrations seems highly unlikely.

In our case-control study (study III) the control subjects were sampled from the Total Population Register were all Swedish residents are included, hence giving all residents the same probability of being sampled.

6.1.2.2 Recall bias

Recall bias occur whenever self-reported exposure-information is used. In the present studies, recall bias was not a problem since information on exposure was retrieved from pre-existing registers and not based on the memory of the study participants.

6.1.2.3 Information bias

Information bias, or misclassification, is a measurement error of the exposure or outcome. Differential misclassification (when the measurement error depends on other variables) may introduce an over- or underestimation of the true association, whereas a non-differential misclassification (when the measurement error is independent of other variables) tend to affect any association towards the null.[117]

Misclassification of outcome

The overall validity in the Swedish hospital discharge register is high, and for most diagnoses the positive predictive values are 85-95%.[113] The positive predictive value for both hip fracture (study I) and myocardial infarction (study II) is close to 100%. The completeness and accuracy of the cancer register is generally high, and over 96% of patients with a cancer diagnose is registered.[116] Therefore the risk of misclassification of outcome is assumed to be small.

Misclassification of exposure

We only assessed fluoride exposure from drinking water, no information on other dietary source for fluoride (such as bottled water, food and dental hygiene products) was available. Drinking water is a major source for fluoride and is estimated to account for 66 to 80% of the total fluoride intake.[7] In Sweden the intake from food is generally low, less than 1 to 6% of the total fluoride intake[27,69], and the consumption of bottled water is limited due to the high tap water quality. The consumption of bottled water was 0.03 L/person/day in 1993 and has since then increased to 0.07 L/person/day in 2014.[126] However, the ingestion of fluoride from dental hygiene products might account for an important part of the total fluoride load in

younger age groups, depending on the fluoride concentration of the product and the amount used and swallowed.[27,34] This measurement error might underestimate any true effect.

Additionally, a non-differential misclassification of exposure cannot be ruled out. Individual fluoride exposure levels were assigned through linkage of parish records to distribution areas of community water works. Moreover, we lack information on actual consumption of tap water and individuals could be categorized as exposed when in fact they might be unexposed and *vice versa*. But according to data from a national health survey in 1999, less than 4% of the Swedish population do not drink tap water at home.[127] All measurement of fluoride levels at each water work is subject to error in the laboratory and when typed into the register. Though, any non-differential misclassification of exposure would be independent of disease and mainly affect the results toward the null.

6.1.2.4 Confounding

Confounding means a mixing of effects. By definition, a confounding factor is associated with the exposure and the outcome, must have an effect, and must be imbalanced between the exposure groups compared.[117] The true association is clouded when confounding is present. Confounding can be handled in many ways, randomization, matching, stratification, restriction and in regression models.[117]

In the present studies we used different methods of handling confounding. In studies I and II potential confounders such as age, sex, calendar period and county of residence were included in the regression models. In study II drinking water hardness was also included in the regression models.

In study III, control subjects were matched to the index person by age at diagnosis and sex. Conditional logistic regression was applied to handle the matching variables. Other potential confounders such as educational level, other cancer and history of bone fractures were included in the regression models.

In studies I and II we also performed additional analysis with stratification for sex, age, geographical area, calendar period for diagnosis. To adjust for the potential confounding effect of hip fracture type in study I, we restricted the analysis to individuals diagnosed with a low-trauma fracture. Additionally, to adjust for the potential confounding effect of fatal and non-fatal myocardial infarction in study II, we restricted the analysis to individuals diagnosed with a fatal or non-fata myocardial infarction. In the matched case control study (study III), we also stratified on sex, different age groups and exposure groups. To adjust for the potential confounding effect of other cancer in study III, we restricted the analysis to individuals without previous other cancer disease. Additionally, to adjust for the potential confounding effect of bone fractures in study III, we restricted the analysis to individuals without previous bone fracture.

In our present studies we did not have information on a number of other potential risk factors such as smoking, alcohol use, body mass index, nutrition, socioeconomic factors etc. The possibility that unmeasured confounding may have influenced the observed findings should be taken into consideration. In study I it is not possible to rule out that the findings of a protective effect of fluoride in the two younger age groups (≤80 years of age) are because of confounding by for example, other dietary factors, smoking, body mass index, alcohol use, and hormone-replacement therapy. In study II, the observed geographical variations in myocardial infarction risk could be explained by other confounding such as socioeconomic factors, hereditary factors, lifestyle, climate, and other compounds in the drinking water. Additionally, in study III cofounding by for example socioeconomic factors, body height, radiation therapy, and genetic factors may have affected the findings and cannot be ruled out.

6.1.2.5 Random error

Random error is referred to as chance, and defined as fluctuations in the estimates that cannot promptly be explained.[117] A finding, both null and positive results can be due to chance, and confidence intervals and p-values roughly estimates the role of chance. The precision is high when the confidence intervals are narrow and the p-value is small, and can be improved by an increased sample size. However, a high degree of precision is not equal to a high degree of validity. Therefore, regardless of sample size and precision, Hill's criteria of causality must be considered (for example strength of the association, consistency, biologic plausibility, dose-response effect).[117]

The large register based cohort in study I and II increased the precision, and resulted in narrow confidence intervals but the main results in the studies were non-significant (confidence intervals including 1). There cannot be ruled out that the observed lack of association is due to chance. In study III the outcome of interest (osteosarcoma) was rare, causing wider confidence intervals and non-significant results. This could possibly be due to lack of power. Furthermore, when performing many sub-analyses the possibility of chance findings increase. Therefore, the findings from sub-analyses in study I-III must be interpreted with caution.

6.1.3 External validity

External validity (generalizability) refers to how relevant findings from the population studied are for populations other than the one studied.[125] Since study I and II were population-based and nation-wide, they are generalizable to the Swedish population. Study III was population-based and nation-wide, and the generalizability in study III is also considered high. Even though the generalizability of the findings (risk estimates) in our studies is restricted to a Swedish population, the findings can provide information for similar populations and settings in other countries.

6.2 FINDINGS AND IMPLICATIONS

6.2.1 Study I

The association between lower fluoride intakes, levels associated with oral health prevention, and bone fracture risk has been assessed in several studies, but the results are inconclusive.[57,68,69] We hypothesized that there would be an association between fluoride level in the drinking water and the risk of hip fracture. We found no such association between fluoride exposure levels ranging between <0.1 and 2.7 mg/L and risk of hip fracture, after adjustments for age, sex, geographical area and calendar period for start of follow-up. Nor did we see an association between fluoride level and the risk of low-trauma osteoporotic fracture. Stratified analyses suggested that fluoride exposure in individuals younger than 80 years of age seemed to be associated with a decreased risk for hip fracture, and no gender difference was detected. However, no clear exposure-response effect was observed. We cannot rule out that unmeasured confounding may have influenced the observed results.

We had no data on other dietary source for fluoride, or data on the use of dental hygiene products containing fluoride. Though, the main source for ingested fluoride is drinking water, and fluoride intake from other sources is limited in our study population. However, we cannot rule out that non differential misclassification of exposure may have affected the results.

Our findings are in line with recent published studies of drinking water fluoride levels around 1.0 mg/L and hip fracture risk, where no association was found.[128-130] Others have found evidence of a decreased risk.[131,132] A previous cohort study[133] using a similar study design as ours, found no association between drinking water fluoride exposure and hip fracture risk among study subjects older than 65 years of age. But, in contrast to our findings of no gender difference, they report an elevated hip fracture risk (RR, 1.44; 95% CI 1.12-1.86) for younger women (<65 years of age) whereas the association for men was the opposite (RR, 0.75; 95% CI 0.51-1.12). However, an important difference between that study and ours is that the cohort consisted of only rural individuals, and the risk patter for hip fracture may be different for rural populations[134,135], and comparisons are difficult to make.

Most of the previous epidemiological evidence that relate drinking water fluoride exposure to hip fracture risk are derived from comparisons of different geographic regions.[136-141] The findings are though difficult to interpret because of analyses based on group data and not on individual data, and the inability to control for confounding factors may distort the true association (ecological fallacy).[142] Moreover, excessive fluoride exposure are shown to have major impact on bone fracture risk[2], but the hazard found in such studies cannot be compared to the much lower exposures that are investigated in our study.

6.2.2 Study II

Studies conducted in fluoride endemic areas have demonstrated an adverse effect from fluoride exposure on the cardiovascular system[90,91,93,94], whereas the epidemiological evidence addressing lower exposure levels is limited. We aimed to investigate the association between drinking water fluoride exposure and the risk of myocardial infarction, and we found no clear evidence of association. Adjustments were made for age, sex, geographical area, calendar period for start of follow-up and drinking water hardness. By restricting the analyses to non-fatal respective fatal myocardial infarction, the findings did not alter. No age, gender, or difference in time period was detected.

As in study I, we had no data on other dietary source for fluoride, or data on the use of dental hygiene products containing fluoride. Though, the main source for ingested fluoride is drinking water, and fluoride intake from other sources is limited in our study population. However, we cannot rule out that non differential misclassification of exposure may have affected the results. Additionally, various trace elements in the drinking water have been investigate in the pathogenesis of cardiovascular disease.[143] We were able to adjust for total water hardness, though adjustments did not alter the results. But data on other geochemical compounds in the drinking water were not available, and we cannot rule out that some minerals could covariate with fluoride and be a source for confounding.

Our main results are in concert with two large cohort studies[144,145], where no association was found between drinking water fluoride exposure and ischemic heart disease. Though, the outcome was death from ischemic heart disease (*i.e.* myocardial infarction) in the previous cohort studies, in contrast to our study where both fatal and non-fatal MI was investigated. Hence, no difference was seen between fatal and non-fatal myocardial infarction in the present study.

Other epidemiological studies have demonstrated an inverse relationship, where very low drinking water fluoride levels were associated with an increased risk of myocardial infarction[89,96-98]. The potential protective effect found on myocardial infarction, has been attributed an indirect association, via oral health improvement[98], but causality cannot be asserted due to bias. Additionally, some of the studies suffer from methodological limitations such as hospital based studies[89], small sample size[89], or low external validity due to only men[89,96,97] or only rural population[98]. We could not confirm these findings. On the contrary, some evidence of a positive association was found between fluoride exposure and myocardial infarction risk in the northern part of Sweden. This association may in part be explained by unmeasured confounding. Moreover, only a small effect size is detected, and in combination with the large sample size and total number of events, we consider that this finding is unlikely to be of significance.

6.2.3 Study III

There is no clear association between fluoride exposure and osteosarcoma risk. According to two systematic reviews, the included studies suffer from methodological deficiencies and lack of homogeneity.[57,69] Specific time windows for exposure has been suggested to be of importance for osteosarcoma development. We hypothesized that there would be an association between fluoride level in the drinking water and the risk of osteosarcoma. No association was found between drinking water fluoride exposure and osteosarcoma risk. Adjustments were made for age, sex, education, other cancer, and bone fractures. Different exposure-times before osteosarcoma diagnosis was studied, as well as different exposure-ages, to evaluate potential important time-windows for exposure.

Our evaluation of different age- and time-specific exposure levels are in contrast with the previous case-control study by Bassin et al.[71] That study found an increased osteosarcoma risk in young boys (<20 years of age) exposed to drinking water fluoride at 6 to 8 years of age, with a peak at 7 years of age, aOR 5.46; 95% CI 1.50-19.90. No consistent association was found among young girls. The study suffered, however, limitations such as selection bias because of difference in referral patterns between cases and controls, and potential recall bias due to retrospective collection of information true interviews. An important difference in our study is the use of information collected in a standardized manner (population-based registries), limiting the possibility of selection and recall bias. A second investigation was conducted based on data from the study by Bassin et al.[71] and additional set of patients from the same population source.[73] In that study the bone fluoride concentrations was investigated, in contrast to fluoride levels in the drinking water. The findings from Bassin et al.[71] could not be confirmed. The use of bone fluoride concentration is an advantage, yet the study suffered from selection bias when controls where other bone cancer patients, *i.e.* if fluoride levels were associated with all type of bone cancer then the study design would not be able to detect any difference in risk.

Our findings are in agreement with findings from a recent large case-control study by Archer et. al.[83] Adjusted unconditional logistic regression analyses stratified by sex and age category (6-8, 9-12, 13-15 and 16+) yielded no evidence of association. However, incomplete residence information and the control selection procedure (controls being other cancer patients), could have introduced bias.

To explore the robustness of our findings, a series of sensitivity analyses were performed. Individuals with other cancer diagnosis prior to index date (osteosarcoma diagnosis) were excluded, and individuals with missing fluoride exposure values were assigned values (impute) based on their existing fluoride values (lowest and highest). The results did not alter in any of the sensitivity analyses.

However, this study has a few important limitations. We lack information on potential confounders such as additional socioeconomic variables and height at diagnosis. Moreover, misclassification of exposure when we only assess fluoride exposure from drinking water,

other dietary source for fluoride (food, beverages, and dental hygiene products) might account for an important part of the total fluoride load in younger age groups.[27,34] And additionally, non-differential misclassification of exposure (*i.e.* individuals categorized as exposed could in fact be unexposed and *vice versa*). We cannot rule out that the above mentioned methodological limitations may have influenced the results.

7 CONCLUSIONS

The results of this thesis add to the body of evidence that ingestion of lower fluoride concentrations (<4 mg/L) is not associated with increased risk of adverse health effects. The major strengths of this thesis is the population-based study design applied in all three studies, the relatively large number of study subjects, which increased the precision, and additionally, that all data were collected prospectively.

Specific conclusions of the three studies:

I. Long term exposure to drinking water fluoride up to 2.7 mg/L seems unlikely to have any important effects on hip fracture risk in Sweden. Additionally, we did not find an association between fluoride level and the risk of osteoporotic (low-trauma) hip fracture. Stratified analyses suggested that fluoride exposure in individuals younger than 80 years of age was associated with a decreased risk for hip fracture. However, no clear exposure-response effect was observed

II. Long term exposure to drinking water fluoride up to 2.7 mg/L seems unlikely to have any important effects on myocardial infarction risk in Sweden. Additional analyses was performed, looking at fatal and non-fatal myocardial infarction. No association was found. We found some evidence of a positive association between fluoride exposure level and risk of myocardial infarction in the northern part of Sweden. This association may in part be explained by unmeasured confounding. Moreover, only a small effect size is detected, and in combination with the large sample size and total number of events, we consider that this finding is unlikely to be of significance.

III. Given the limitations, no association was found between drinking water fluoride levels up to 2.7 mg/L and osteosarcoma risk in Sweden. No age-, time-, or sex-specific associations were detected.

8 FUTURE PERSPECTIVES

Throughout this thesis, only the fluoride content of the drinking water was assessed and evaluated. Even though, drinking water is the biggest contributor to fluoride exposure, future research projects investigating the association between health effects and ingested fluoride, should also include fluoride from other sources, such as food, beverages, and ingested fluoride from dental hygiene products.

Ingested fluoride is mainly eliminated and excreted via the kidneys. Future studies on the association between ingested fluoride and health risk in individuals with renal impairments, in whom more fluoride may be retained than in healthy individuals.

Future studies on the effect of ingested fluoride on human intelligence. Human studies of ingested fluoride have been reporting adverse behavioral and cognitive effects. However, the studies suffer from methodological limitations, and the exposure levels are high and not applicable to Swedish populations.

Future studies on the association between ingested fluoride and health risks in a bottled-fed population, which might have ingested a higher amount of fluoride than a breast-fed population.

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